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सत्यमेव जयते

GOVERNMENT OF INDIA
MINISTRY OF COMMERCE & INDUSTRY,
PATENT OFFICE, DELHI BRANCH,
W - 5, WEST PATEL NAGAR,
NEW DELHI - 110 008.

BEST AVAILABLE COPY

I, the undersigned, being an officer duly authorized in accordance with the provision of the Patent Act, 1970 hereby certify that annexed hereto is the true copy of the ***Application*** *and* ***Complete Specification*** *filed in connection with Application for Patent No.**1187/Del/02** dated **26th November 2002.***

Witness my hand this 9th Day of September 2003.

(S.K. PANGASA)
Assistant Controller of Patents & Designs

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

1119 DEL 02

27 NOV 2002

FORM 1

26 NOV 2002

THE PATENTS ACT, 1970
(39 OF 1970)
APPLICATION FOR GRANT OF A PATENT

1. I, (a) the **Director General**

   (b) Defence Research & Development Organisation
   Ministry of Defence, Govt of India,
   B-341, Sena Bhawan, DHQ P.O.
   New Delhi – 110011

   (c) an Indian National

2. hereby declare –

   (a) that I am in possession of an invention titled :
   'A Process for the Preparation of an Agglutination Reagent for Rapid Detection of Typhoid'

   (b) that the Provisional/Complete Specification relating to this invention is filed with this application.

   (c) that there is no lawful ground of objection to the grant of a patent to me.

3. further declare that the inventor(s) for the said invention are :-

(a) Names Principal Names first)	(i)	GANGA PRASAD RAI
	(ii)	GAURI SHANKAR AGARWAL
	(iii)	SHRI KRISHNA SHARMA
	(iv)	DEVENDRA KUMAR JAISWAL
	(v)	KRISHANAMURTHY SHEKHAR
	(vi)	KAJAL ARORA
	(vii)	VIJAY KUMAR CHAUDHARY

Address : For (I) to (v) Defence Research & Development Establishment, Jhansi Road, Gwalior - 474 002
For (vi) & (vii) Deptt of Biochemistry, Delhi University Smooth campur, Benito Juares Road, New Delhi - 110 002

(c) Nationality : All Indian Nationals

4. I, claim the priority from the application(s) filed in convention countries, particulars of which are as follows:

(a) Name of the country	: Nil
(b) Application No.	: Nil

(c) Date of Application : Nil
(d) Applicant in Convention Country : Nil
(e) Title of Invention in the Convention Country : Nil

5. I state that the said invention is an improvement in or modification of the invention, the particulars of which are as follows and of which I am the applicant/patentee:

(a) Application No. or Patent No. : Nil
(b) Date of Application or Date of Patent : Nil

6. I state that the application is divided out of my application, the particulars of which are given below and pray that this application deemed to have been filed on ____NA____ under Section 16 of the Act.

(a) Application No including Published Sr No, if any : Nil

(b) Date of filing of Provisional/Complete specifications : Nil

7. That I am the assignee of the true and first inventors.

8. That my address for service in India is as follows: M/s L.S.DAVAR & CO., Patent & Trademarks Attorney, 5/1, (First Floor), Kalkaji Extension, New Delhi – 110019.

9. Following declaration was given by the inventors :

We the true and first inventors for this invention declare that the applicant herein is our assignee

(a) Names (Principal Names first)
(i) GANGA PRASAD RAI
(ii) GAURI SHANKAR AGARWAL
(iii) SHRI KRISHNA SHARMA
(iv) DEVENDRA KUMAR JAISWAL
(v) KRISHNAMURTHY SEKHAR
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(b)Address : For (i) to (v) : Defence Research & Development Establishment, Jhansi Road, Gwalior – 474 002, India
For (vi) & (vii) : Department of Biochemistry, Delhi University South Campus, Benito Juarez Road, New Delhi – 110 002.

(c) Nationality : All Indian Nationals

Signatures of Inventor(s)

(Full name in brackets)

(i) GANGA PRASAD RAI

(ii) GAURI SHANKAR AGARWAL

(iii) SHRI KRISHNA SHARMA

(iv) DEVENDRA KUMAR JAISWAL

(v) KRISHNAMURTHY SEKHAR

(vi) KAJAL ARORA

(vii) VIJAY KUMAR CHAUDHARY

10. That to the best of my knowledge, information and belief, the fact and matters stated herein are correct and that there is no lawful ground of objection to the grant of patent to me on this application.

11. Following are the attachment with the application:

(a) ~~Provisional~~/Complete specification (in triplicate)

(b) Drawings (3 copies). – Nil
(c) Formal Application (in triplicate)
(d) Form – 3
(e) Fee of Rs. 5000/-

I request that a patent may be granted to me for the said invention.

Dated this day of

(Avinash Kumar)
Deputy Director (IPR)
for Director General
Defence Research & Development Organisation

To
The Controller of Patents
The Patent Office,
New Delhi.

THE PATENTS ACT, 1970

COMPLETE 1 1 DEL 02

SPECIFICATION

26 NOV 2002

26 NOV 2002

SECTION 10

TITLE

A PROCESS FOR PREPARATION OF AN AGGLUTINATION REAGENT FOR RAPID DETECTION OF TYPHOID

APPLICANT

DIRECTOR GENERAL
Defence Research & Development Organisation, Ministry of Defence, Government of India, B-341, Sena Bhawan, DHQ P.O., New Delhi-110 011, India, an Indian National

The following specification particularly describes and ascertains the nature of this invention and the manner in which it is to be performed.

FIELD OF INVENTION

The present invention relates to a process for the preparation of an agglutination reagent for rapid and early detection of *Salmonella typhi* in serum.

PRIOR ART

Typhoid is an endemic febrile disease caused by *Salmonella typhi*. Typhoid is a major concern of public health. The organism usually enters the body by consumption of contaminated food or water and penetrates the intestinal wall. After that it multiplies and enters blood stream within 24-72 hours resulting in enteric fever and bacteremia. After an incubation period of 10 to 14 days, early symptoms of typhoid, like headache, fever, loss of appetite, bradycardia, splenomegaly etc. appear. Typhoid is diagnosed either by blood culture or by detection of its antigens or by the detection of its antibodies in the blood.

One of the most adapted methods for diagnosing the typhoid fever is the performance of "Widal test", a serological test based on the detection of antibodies in the blood. This test is based on the fact that antibodies against typhoid, remain in the blood of infected person, bind to the bacteria and results in the clumps formation which is referred as "Widal Agglutination".

One of the limitation of the widal test is that the test is not specific as it cross reacts with other febrile organisms and many organisms of family Enterobacteriaceae.

Another limitation of the widal test is that, as typhoid is an endemic disease hence there always exist some background level of antibody in the endemic areas. Hence it becomes necessary to determine the cut-off titre for each region to rule out the possibility of diagnosis as false positive.

Yet another limitation of the widal test is that it gives positive results only after one or two weeks of the onset of fever.

Still another limitation of the widal test is that test it is to be performed on paired serum samples taken at an interval of at least one week apart because single widal test is elusive and inconclusive.

Further limitation of the widal test is that the antibiotic administration in the early phase of infection, inhibits the development of the antibody and hence test may give false negative result.

Still further limitation of the widal test is that TAB vaccinated normal healthy persons give false positive reaction in widal test due to presence of circulating antibody against vaccine in human system.

Another limitation of the widal test is that it gives indirect evidence of typhoid infection.

Further limitation of the widal test is that the test has low sensitivity and low specificity.

Other technique known for diagnosis of typhoid is based upon isolation and identification of causative agent. This procedure is termed as golden standard.

In this technique *Salmonella typhi* is isolated from blood and identified by microscopic and biochemical tests. However, this technique has many limitations.

One limitation of the above technique is that it is time consuming as it requires long period of incubation from 3 days to 14 days and also requires elaborate laboratory facilities.

Another limitation of the above technique is that for its performance large quantity of blood sample (10 ml/patient) is required.

Yet another limitation of the above technique is that it needs large volume of culture medium i.e. 100 ml (10 times of blood sample).

Still another limitation of the above technique is its low sensitivity (40 to 60%), as there are very few organism in circulation, as low as 1/ml which leads to false negative results.

Further limitation of above method is that bacterial growth in culture is inhibited by serum bactericidal agents, present in blood which may lead to false negative results.

Still further limitation of blood culture is that antibiotics treatment during early phase of infection may inhibit bacterial growth in culture which may give false negative results.

Other known techniques such as Radioimmunoassay (RIA), Enzyme - linked immunosorbent assay etc. are based on detection of circulating antigen in the body fluids, but these techniques have many limitations.

One limitation of these techniques is that they require sophisticated and elaborate laboratory facilities.

Another limitation of RIA is that it requires radioactive material which is health hazard and also needs trained personnel to handle the radioactive material.

Still further limitation of above techniques is that reagents are expensive.

Further limitation of these techniques is that minimum 4-5 hours are required to perform the tests.

OBJECT OF THE INVENTION

The primary object of the present invention is to provide a process for the preparation of an agglutination reagent for rapid and early detection of *S.typhi* in serum samples of suspected typhoid patients.

Another object of the present invention is to provide a process for the preparation of an agglutination reagent wherein the proposed reagent enables diagnosis of typhoid within 3 minutes after collection of serum samples.

Yet another object of the present invention is to provide a process for the preparation of an agglutination reagent wherein the serum sample required for the diagnosis of typhoid disease is as small as 20 µl.

Further object of the present invention is to provide a process for the preparation of an agglutination reagent wherein the reagent enables the detection of typhoid bacteria by simple latex agglutination technique.

Still further object of the present invention is to provide a process for the preparation of an agglutination reagent wherein the reagent enables specific identification of *Salmonella typhi* antigen in serum samples of suspected typhoid patients.

Yet further object of the present invention is to provide a process for the preparation of an agglutination reagent wherein the reagent enables the diagnosis of typhoid in the early stages of infection even within one or two days after the on set of the fever.

Another object of the present invention is to provide a process for the preparation of an agglutination reagent wherein the reagent is highly sensitive.

Yet another object of the present invention is to provide a process for the preparation of an agglutination reagent which enables diagnosis of the disease in field conditions as it does not require any equipment or laboratory facility.

Still further object of the present invention is to provide a process for the preparation of an agglutination reagent that does not require any specially trained personnel to perform the test.

Yet further object of the present invention is to provide a process for the preparation of an agglutination reagent which enables the diagnosis of those patients who have been administered with antibiotics resulting in blood culture isolation as negative.

DESCRIPTION OF PROCESS

According to the preferred embodiment of the present invention, the agglutination reagent is prepared by a process comprising of following steps:

(a) Preparation of antibody (immunoglobulins):

Flagellin gene sequence specific to *Salmonella typhi* is cloned and expressed by recombinant DNA technology. The expressed recombinant protein is purified by affinity chromatography. Hyper immune sera against this recombinant protein is raised in rabbit. Immunoglobulin fraction of hyper immune sera is separated by ammonium sulphate precipitation. The precipitated immunoglobulins are suspended in 50mM phosphate buffer (pH 7.2), dialysed and protein content determined.

b) Preparation of Latex Particles suspension:

1% carboxylated latex particles of size 0.88 to 0.90 µm and 40mM 2-N Morphilinoethane sulphonic acid (MES) buffer (pH 5.5-6.0) are taken in a preferred ratio of 1:1 in a tube. They are mixed on a vortex mixer for around 60 seconds and centrifuged at 10,000 rpm for 10-12 minutes at about 4°C. The latex particles are further washed twice in 20mM MES buffer of pH 5.5 by mixing on vortex mixer for around 60 seconds, followed by centrifugation at 10,000 rmp for 10-12 minutes at about 4°C. After the final wash, the latex particles are suspended in 20mM MES buffer of pH 5.5 and the volume is made up equal to the starting volume of the latex particles. The suspension is then sonicated by a tip sonicator at about 5 watts for 60-120 seconds, preferably 90 seconds. To this suspension freshly prepared solution of 0.1 M1-ethyl-3(3-dimethyl-amino-propyl) carbodimide hydrochloride (EDC) in 20mM MES buffer (pH 5.5) taken in the preferred ratio of 1:1, is added drop wise, while the solution is slowly vortexed. The tube is rotated slowly end-over-end for about 3 hours at a

temperature of 20-25°C. It is then washed thrice with 20mM MES buffer (pH 5.5) at 10,000 rpm for 10-12 minutes at a temperature of about 4°C. The latex particles are resuspended in MES buffer (20mM, pH5.5) and sonicated for 60-120 seconds by a tip sonicator at 5 watts.

(c) Coating of Latex Particles with Antibody (immunoglobulins):

To the suspension of latex particles prepared in step(b), 0.6-1.0 mg preferably 0.8 mg per ml of the suspension, immunolobulins prepared in step (a) are added. The whole mixture is then rotated end-over-end for 18-20 hours at a temperature of 20-25°C. The coating reaction is stopped by addition of 1M glycine (pH 11.0) taken in quantity of 0.06ml per ml of solution of immunoglobulin coated latex particles. Rotation is continued for about 30 minutes at a temperature of 20-25°C. The coated latex particles are pelleted out by centrifugation at 10,000 rpm for 10-12 minutes at a temperature of about 4°C. The pellet is washed thrice with washing buffer (50mM glycine, pH8.5; 0.03% triton X-100 and 0.05% sodium azide) at 10,000 rpm for 10-12 minutes at a temperature of about 4°C. Finally the washed coated latex particles are resuspended in storage buffer (50mM glycine, pH8.5; 1.0% bovine serum albumin; 0.03% triton X-100; 0.1% sodium azide and 0.01% thiomersol) to a final concentration of 1% and sonicated by a tip sonicator for around 60 seconds at about 5 watts and stored at 4°C.

METHOD OF USE

(a) Take 20-40µl (1 to 2 drops) of test serum, positive and negative controls at three distinct places on a glass slide
(b) Add 10-2µl (1-2 small drops) of latex reagent to test serum, positive and negative controls
(c) Mix the reactants with separate wooden sticks carefully to avoid any intermixing of reactant placed at separate places and rotate the slide for 1-2 minutes.

A positive reaction is indicated by the development of an agglutination within 1-2 minutes of mixing the reagent with the test sample and positive control, showing clearly visible clumping of the particles. The speed of appearance and quality of agglutination depends on the strength of the antigen present, varying from large clumps which appear within a few seconds of mixing, to small clumps which develop rather slowly. In negative reaction the reagent does not agglutinate and the cloudiness or the turbid nature remains substantially unchanged throughout the test.

Laboratory studies on the reliability of proposed agglutination reagent for rapid detection of *Salmonella typhi* in typhoid patient serum is performed with the laboratory strains of Salmonella typhi; and culture proven and widal positive serum samples collected from suspected cases of typhoid; and with serum samples of apparently normal healthy individuals. The result indicate 93.00% sensitivity and 98.00% specificity.

The present invention will now be illustrated with a working example which is intended to be illustrative example and is not intended to be taken restrictively to imply any limitation on the scope of the present invention.

WORKING EXAMPLE

Flagellin gene sequence specific to *Salmonella typhi* was amplified by polymerase chain reaction (PCR) using gene specific primers. Amplified PCR product was cloned in Glutathione-S-transferase (GST) vector-and-later-expressed. The expressed protein was purified by GST affinity column chromatography. The protein content of the purified product was determined by Bradford method. Hyper immune serum against this protein was raised in rabbit. Immunoglobulins fraction of hyper immune sera was separated by ammonium sulphate precipitation. The precipitated immunoglobulins were suspended in 1.0 ml PB (50 mM, pH 7.2), dialysed and protein content determined. 1.0 ml of 1% carboxylated latex particles and 1.0 ml of 40 mM MES buffer (pH 5.5 - 6.5) were taken in 2.0 ml microcentrifuge tube. Then they were mixed on vortex mixer for 60 seconds and centrifuged at 10,000 RPM for 10-12 minutes at a temperature of 4°C. The latex particles were further washed twice in 2.0 ml of 20mM MES buffer (pH 5.5) by mixing on vortex mixer for 60 seconds and centrifugation at 10,000 RPM for 10-12 minutes at a temperature of 4°C. Following the final wash, the latex particles were suspended in 1.0 ml MES buffer (20mM, pH5.5) and sonicated by a tip sonicator at 5 watts for 60-120 seconds. Later 1.0 ml of freshly prepared solution of 0.1 M 1-ethyl-3-(3-dimethylaminopropyl) carbodimide hydrochloride (EDC) in MES buffer (20mM, pH5.5) was added drop wise while the solution was slowly vortexed . Then the tube was rotated slowly end-over-end for 3 hours at a temperature of 20-25°C followed by washing three times with MES buffer (20mM, pH 5.5) at 10,000 RPM for 10-12 minutes at a temperature of 4°C. The latex particles were resuspended in 0.7 ml MES buffer (20 mM, pH 5.5) and sonicated for 60-120 seconds by a tip sonicator at 5 watts. 0.8 mg of immunoglobulins were added to latex particles and volume was made up to 1.0 ml with MES buffer (20mM, pH 5.5). This was then rotated end-over-end for 18-20 hours at a temperature of 20-25°C. The coating reaction was then stopped by addition of 0.06 ml of 1M glycine (pH 11.0). The rotation was continued for 30 minutes at a temperature of 20-25°C. The coated latex particles were pelleted out by centrifugation at 10,000 RPM for 10-12 minutes at a temperature of 4°C. The pellet was washed thrice with 2.0 ml of washing buffer (50 mM glycine, pH 8.5, 0.03% triton X-100 and 0.05% sodium azide) at 10,000 RPM for 10-12 minutes at a temperature of 4°C. The washed coated latex particles were resuspended in storage buffer (50mM glycine, pH 8.5, 1.0% bovine serum albumin, 0.03% triton X-100, 0.1% sodium azide and 0.01% thiomersol) to a final concentration of 1% and sonicated with tip sonicator for 60 seconds at 5 watts and stored at 4°C.

It is to be understood that the present invention is susceptible to modifications, changes and adaptations by those skilled in the art. Such modifications, changes, adaptations are intended to be within the scope of the present invention which is further set forth under the following claims:-

WE CLAIM:

1. A process for the preparation of an agglutination reagent for rapid and early detection of typhoid, comprising in the steps of:

   (a) preparing *Salmonella typhi* specific antibody
   (b) preparing latex particles suspension
   (c) coating said latex particles with the antibody

2. A process as claimed in claim 1 wherein Flagellin gene sequence specific to *Salmonella typlhi* is cloned and expressed by recombinant DNA technology.

3. A process as claimed in claim 2 wherein the expressed recombinant protein is purified by affinity chromatography.

4. A process as claimed in claim 3 wherein the hyper immune sera against the recomninant protein is raised in rabbit.

5. A process as claimed in claim 4 wherein the immunoglobulin fraction of hyper immune sera is separated by ammonium sulphate precipitation.

6. A process as claimed in claim 1 wherein the precipitated immunoglobulins are suspended in phosphate buffer dialysed and the protein content is determined.

7. A process for the preparation of an agglutination reagent for rapid and early detection of typhoid as claimed in claim 1 wherein 1% carboxylated latex particles of size 0.88 to 0.90 μm and 40mM 2-N Morphilinoethane sulphonic acid (MES) buffer of pH 5.5 to 6.0 are taken in a preferred ratio of 1:1.

8. A process for the preparation of an agglutination reagent for rapid and early detection of typhoid as claimed in claim 1 wherein the suspension of latex particles in MES buffer is mixed on a vortex mixer for around 60 seconds and centrifuged at 10,000 rpm for 10-12 minutes at about 4°C.

9. A process for the preparation of an agglutination reagent for rapid and early detetion of typhoid as claimed in claim 1 wherein the latex particles are further washed twice with 20mM MES buffer of pH 5.5 at 10,000 rpm for 10-12 minutes at about 4°C.

10. A process for the preparation of an agglutination reagent for rapid and early detection of typhoid as claimed in claim 1 wherein the washed latex particles are suspended in 20mM MES nuffer of pH 5.5 and the volume is made up as equal to the starting volume of latex particles.

11. A process for the preparation of an agglutination reagent for rapid and early detection of typhoid as claimed in claim 1 wherin the suspended latex particles are sonicated by a tip sonicator at anout 5 watts for 60-120 seconds preferably 90 seconds.

12. A process for the preparation of an agglutination reagent for rapid and early detection of typhoid as claimed in claim 1 wherein freshly prepared solution of 0.1 M 1-ethyl-3 (3-dimethyl-amino propyl) carbodimide hydrochloride (EDC) in 20mM MES buffer of pH 5.5, in a preferred ratio of 1:1 is added drop wise to the latex prticles suspension, while the suspension is slowly vortexed.
13. A process for the preparation of an agglutination reagent for rapid and early detection of typhoid as claimed in claim 1 wherein the latex particles with EDC solution are rotated slowly end-over-end for about 3 hours at a temperature of 20-25°C and washed thrice with 20mM MES buffer (pH 5.5) at 10,000 rpm for 10-12 minutes at a temperature of about 4°C.
14. A process for the preparation of an agglutination reagent for rapid and early detection of typhoid as claimed in claim 1 wherein the latex particles are resuspended in MES buffer (20mM, pH5.5) and sonicated by a tip sonicator for 60-120 seconds at about 5 watts.
15. A process for the preparation of an agglutination reagent for rapid and early detection of typhoid as claimed in claim 1 wherein to the latex particle suspension prepared in step (b), 0.6-1.0 mg preferably 0.8 mg per ml of the immunoglobulins prepared in step (a) are added.
16. A process for the preparation of an agglutination reagent for rapid and early detection of typhoid as claimed in claim 1 wherein the suspension of latex particles and immunoglobulins is rotated end-over-end for 18-20 hours at a temperature of about 20-25°C.
17. A process for the preparation of an agglutination reagent for rapid and early detection of typhoid as claimed in claim 1 wherein the coating reaction is stopped by 1M glycine (pH 11.0) taken in quantity of 0.06 ml per ml of solution of immunglobulin coated latex particles.
18. A process for the preparation of an agglutination reagent for rapid and early detection of typhoid as claimed in claim 1 wherein the coated latex particles are pelleted out by centrifugation at 10,000 rpm for 10-12 minutes at a temperature of about 4°C.
19. A process for the preparation of an agglutination reagent for rapid and early detection of typhoid as claimed in claim 1 wherein the pellet of coated latex particles is washed thrice with washing buffer comprising of 50mM glycine, pH8.5; 0.03% triton X-100 and 0.05% sodium azide; at 10,000 rpm for 10-12 minutes at a temperature of about 4°C.
20. A process for the preparation of an agglutination reagent for rapid and early detection of typhoid as claimed in claim 1 wherein the washed and coated latex particles are suspended in storage buffer comprising of 50mM glycine pH8.5; 1.0% bovine serum albumin; 0.03% triton X-100; 0.1% sodium azide and 0.01% thiomersal, to a final concentration of 1%.
21. A process for the preparation of an agglutination reagent for rapid and early detection of typhoid as claimed in claim 1 wherein the 1% suspension of coated latex particles is sonicated by a tip sonicator for around 60 seconds at about 5 watts and stored at 4°C.
22. A process for the preparation of an agglutination reagent as substantially described and illustrated herein.

DATED THIS @@22nd DAY OF NOVEMBER, 2002

(G.S.DAVAR)
OF L.S.DAVAR & CO.,